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Alterations in Bidirectional Transmembrane Calcium Flux Occur Without Changes in Protein Kinase C Levels in Rat Aorta During Sepsis

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A depression in aortic contractility has been previously demonstrated in rat intraperitoneal sepsis and during endotoxemia. In this study, we determined whether the mobilization of extracellular calcium (using ^{45}Ca) and the release of intracellular calcium are altered in septic rat aorta when compared to sham-operated controls. The concentration of protein kinase C was also determined by using [^3H] phorbol-12,13-dibutyrate (PDBu). We found that calcium influx was unaltered under basal conditions but that the ability of norepinephrine (NE) to augment influx was significantly depressed ($P < .05$; [control vs. septic, $572 (\pm 54 \text{ SE})$ vs. $428 \pm 30 \mu\text{mol Ca}^{2+}/\text{kg dry wt. aorta}$). Calcium influx stimulated by high K^+ was unchanged in aortae between control and septic animals. In the presence of NE, calcium efflux (an indirect measurement of intracellular calcium release) was significantly diminished ($P < .001$) in aortae from septic rats. The concentration of aortic protein kinase C as assessed by PDBu binding sites was unaltered in septic rats when compared with controls. In conclusion, we found that during sepsis α_1 -adrenergic receptor activation of both calcium influx and efflux by NE is decreased; these alterations could be related to the depressed aortic contractility observed in sepsis.

Key words: calcium influx, calcium efflux, norepinephrine

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These findings were presented at the First International Shock Congress, 1987 and published in preliminary form [28].

The opinions and assertions contained herein are the private ones of the authors and should not be construed as reflecting the views of the U.S. Navy, the naval service at large, the Department of Defense, or the Defense Nuclear Agency.

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INTRODUCTION

Human sepsis and septic shock are frequent causes of death in clinical medicine. Septic shock is characterized by peripheral vasodilation with a decreased systemic vascular resistance and hypotension [1-3]. In an attempt to increase the vascular resistance and elevate blood pressure, clinicians usually administer catecholamines and catecholamine derivatives. However, a diminished peripheral vascular responsiveness to catecholamines is commonly observed [4], thus making this approach frequently ineffective.

Alterations in vascular contractility may either involve extrinsic factors (prostaglandins, opioid peptides, etc.) or may be intrinsic to vascular smooth muscle. During endotoxemia and sepsis, previous investigators have demonstrated a diminished ability of NE to induce contraction in rat aorta [see ref. 3 for review]. We previously demonstrated a decrease in the number of aortic α_1 -adrenoceptors and a depressed aortic phosphoinositide (PI) metabolism in this rat model of chronic sepsis [5]. Since we have shown that activation of the α_1 -adrenoceptors with NE results in a stimulation of PI metabolism [6,7], it appears that signal transduction involving α_1 -adrenoceptors is altered in aortae from septic rats.

In this study we determined whether calcium mobilization was altered in aortae from septic rats during the stimulation of α_1 -adrenergic receptors by NE. Calcium influx through calcium channels and calcium efflux, an indication of intracellular calcium release by the sarcoplasmic reticulum [8], were measured in the rat aortae. Both processes in rat aorta are stimulated by the activation of α_1 -adrenoceptors [9]. In addition, we measured the levels of protein kinase C as determined by PDBu binding sites in aortae from septic rats. Activated protein kinase C has been shown to phosphorylate smooth muscle myosin light chain [10,11] as well as alter calcium influx in vascular smooth muscle [12,13] and induce rat aortic contraction [14]. We have also shown that activation of protein kinase C inhibits PI hydrolysis [7], which could explain, in part, the diminished PI turnover observed in the rat aorta during sepsis [5].

MATERIALS AND METHODS

Chronic Rat Sepsis Model

Male Sprague Dawley rats (250-300 g) were obtained from Taconic Farms, Germantown, NY. Control and experimental animals were randomly subjected to sham surgery or cecal ligation with two-hole puncture, respectively, as previously described [15,16]. Twenty-four hours after surgery the surviving animals were killed by decapitation. In our laboratory the model has a 20-30% mortality rate. Surviving animals displayed signs of sepsis originally described by Wichterman et al. [15] including piloerection, a bloody discharge from the nose and mucous membrane, bloody diarrhea, and lethargy.

^{45}Ca Influx

Calcium influx was measured as described by Meisheri and van Breemen [17] with minor modifications [13]. Briefly, 4-mm thoracic aortic rings were incubated at 37°C for 45 min in oxygenated physiological saline (PSS) of the following composition (in mM): NaCl (140), MgCl_2 (1), CaCl_2 (1.5), KCl (4.6), glucose (10), HEPES buffer (5), pH 7.4 (buffer A). The rings were transferred to PSS (37°C) containing ^{45}Ca (2 $\mu\text{Ci/ml}$) and various experimental protocols as described in figure legends. The rings were then washed in ice-cold PSS for 30 min and placed in a hypotonic 5.0 mM EDTA solution overnight at

room temperature. The aortic rings were removed, dried overnight at 90°C, and weighed. The radioactivity in the hypotonic solution was then detected by liquid scintillation counting.

⁴⁵Ca Efflux

Calcium efflux was measured in rat aorta by using the procedure of Chiu et al. [9] with minor modifications. Briefly, 4-mm aortic rings were incubated at 37°C for 30 min in oxygenated low-calcium buffer A (0.2 mM instead of 1.5 mM CaCl₂). The rings were then loaded with ⁴⁵Ca for 90 min by using low-calcium buffer A containing 4 μCi/ml of ⁴⁵Ca. The rings were placed in ice-cold low calcium buffer A for 40 min with a change in solution at 10 min. The rings were then transferred to buffer A (1.5 mM CaCl₂) at 37°C for a series of washout procedures wherein the buffer A solutions were changed at 5-, 15-, 20-, and 25-min intervals. A final concentration of 10 μM NE was added to the experimental solution at the start of the 20-min washout period, where an increase in ⁴⁵Ca release was observed (see Fig. 1). The radioactivity was measured in the washout solution, and the aorta was placed in hypotonic 5.0 mM EDTA solution overnight at room temperature. The aortic rings were removed, dried overnight at 90°C, and weighed. The radioactivity in the hypotonic solution was then counted. The calcium efflux was expressed as cpm ⁴⁵Ca released into the washout solution per mg dry weight of aorta (see Fig. 1).

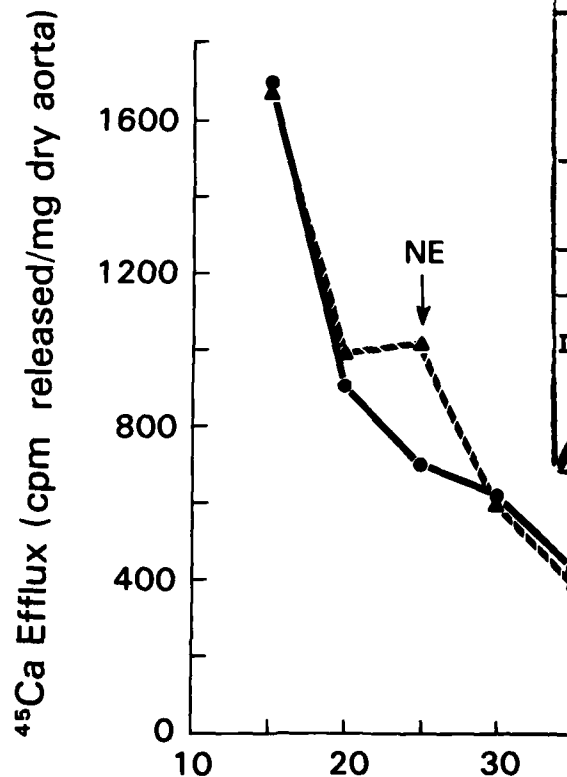


Fig. 1. A typical calcium efflux experiment from rat aortic rings expressed as cpm ⁴⁵Ca released per mg dry weight of aorta vs. time. At 20 min 10 μM of NE was added to one of the aortic rings (▲), resulting in an increase in calcium release. ● represents calcium efflux without any norepinephrine.

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[³H]-Phorbol-12,13-Dibutyrate Binding

PDBu binding was measured as described by Sando and Young [18]. In brief, five aortae were homogenized in 5 ml of homogenization buffer (20 mM Tris-Cl, 0.1 mM phenylmethylsulfonylfluoride, 0.1% beta-mercaptoethanol, 1 mM EGTA, pH 7.40); 100 μ l of homogenate was incubated with 0.5 ml of binding buffer (20 mM Tris-Cl, 2 mM CaCl₂, 10 mM MgCl₂, pH = 6.40, 0.1 mg/ml phosphatidylserine) for 90 min at 22°C. PDBu binding sites were harvested on Whatman GF/B filters with a Brandel Cell Harvester followed by three 5-ml washes with ice-cold 20 mM Tris-Cl (pH 7.40).

Statistical Analysis of the Data

All data reported here represent the mean \pm standard error (SEM). The Student's t-test was used for unpaired samples. The Bonferroni inequality was used for simultaneous multiple comparisons [19].

RESULTS**Ca²⁺ Influx**

Calcium influx through calcium channels was measured in aortic rings from control and septic rats. We previously showed this to involve a nitrendipine-sensitive process [13]. Under basal conditions there was no difference in calcium influx. The aortic rings were next stimulated by 10 μ M NE, a concentration which produced maximal aortic isometric contraction [20] and which in preliminary experiments resulted in maximal influx (not

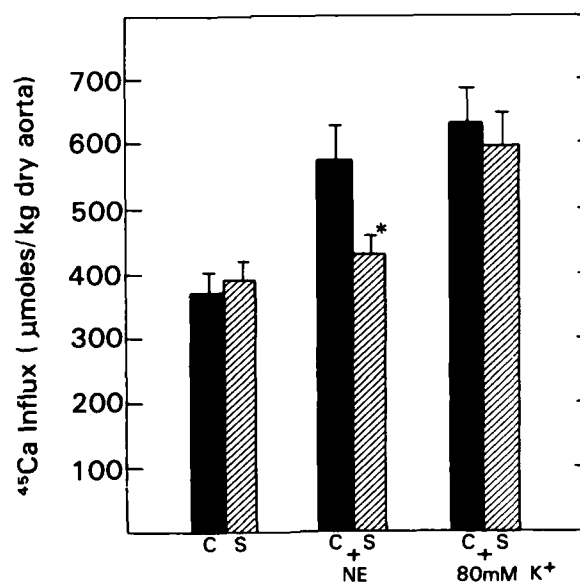


Fig. 2. Calcium influx in control (C) and septic (S) aortic rings. Calcium influx was measured at 5-min incubation time under basal conditions, as well as stimulatory conditions using 10 μ M NE and 80 mM K⁺. Values are mean \pm SEM for 18–34 rings. * P < .05 vs. control + NE values. A depression in the NE activation of calcium influx over the basal levels was also observed in aortae from septic rats at incubation times of 10 and 15 min. No difference was observed in the high K⁺-stimulated calcium influx between control and septic rats at 10-min incubation time.

shown). We found that the NE-stimulated calcium influx was significantly depressed ($P < .05$) when compared to control values (Fig. 2). Comparison of NE stimulation of calcium influx with the basal levels of calcium influx showed an 88% increase in controls and only a 13% increase in aortae from septic rats. When calcium influx was stimulated by high K^+ , there was no significant difference between control and septic animals.

Ca^{2+} Efflux

Calcium efflux was next determined to investigate changes in intracellular calcium release. We found that under basal conditions there was a small but significant ($P < .05$) decrease in calcium efflux in aortic rings from septic animals when compared to controls (Fig. 3). During activation with NE, the calcium efflux was significantly reduced by 33% ($P < .001$ vs. controls) in aortae from septic rats.

Protein Kinase C in Aortae From Septic Rats

As is seen in Table I, there was no difference in total protein kinase C (as measured by PDBu binding) in aortae from septic and control rats.

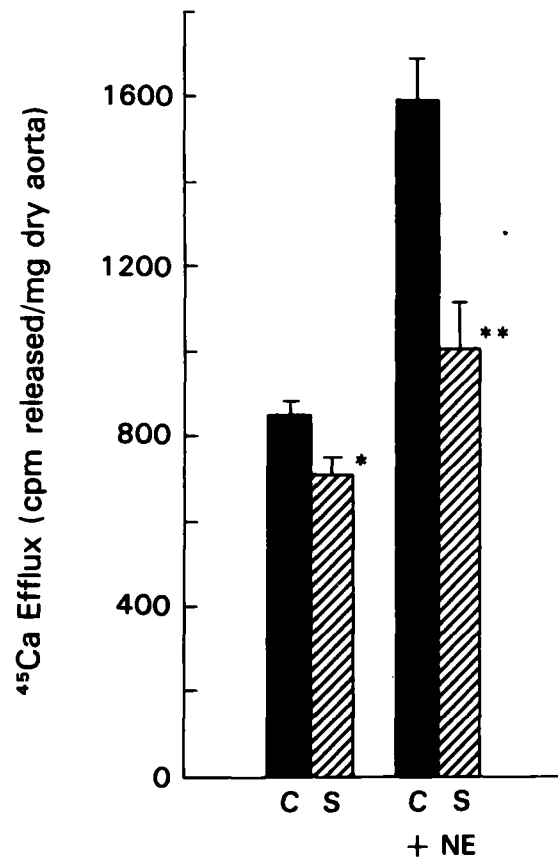


Fig. 3. Calcium efflux in control (C) and septic (S) aortic rings. Calcium efflux was measured in the presence and absence (basal) of $10 \mu M$ NE. Values are mean \pm SEM for 14–16 rings. * $P < .05$ vs. control values; ** $P < .001$ vs. control + NE values.

TABLE I. [³H]phorbol-12,13-Dibutyrate Binding to Aortic Homogenate From Control and Septic Rats^a

	Total PDBu binding site (dpm/mg)
Control	34,000 ± 3,500
Septic	32,500 ± 4,250

^aValues are mean ± SEM for three different experiments. Data obtained are described in Materials and Methods. There was no significant difference between control and septic preparations.

DISCUSSION

The findings in this study indicate significant alterations in α_1 -adrenergic receptor-mediated mobilization of extracellular calcium and the release of intracellular calcium in aortae from septic rats. Calcium influx was depressed by 25%, while calcium efflux was reduced by 33% when compared with control values. At the same time, we found that the amount of protein kinase C as assessed by PDBu binding sites was unaltered in aortae from septic rats.

The decrease in calcium mobilization across the calcium channels could be related to the observed depression in aortic contractility during sepsis. This effect could be caused directly by endotoxin or by other circulatory factors such as lymphokines or endothelium-releasing factor(s). We previously showed that the activation of protein kinase C by a phorbol ester increases the calcium influx in rat aorta [13] as well as altering the ability of vasoactive agents to activate PI hydrolysis [21,22]. Since levels of protein kinase C were unchanged it is unlikely that changes in protein kinase C caused the observed change; a more likely explanation is the decrease in receptor levels in rat aorta, as we previously discovered [5]. We also found that PI metabolism was depressed in the rat aorta during sepsis [5], thus creating the possibility of decreased activation of protein kinase C by diacylglycerol (DAG), one of the released products of PI metabolism [23]. Future studies using specific antibodies against protein kinase C could verify this distinction between activation of protein kinase C (i.e., by DAG) and the amount of the enzyme.

The decreased calcium efflux seen in sepsis suggests that intracellular calcium release from the sarcoplasmic reticulum may be depressed in response to NE. This depression could be related to a decrease in inositol-1,4,5-trisphosphate (IP_3), another product of PI metabolism which has been shown to cause calcium release from sarcoplasmic reticulum in vascular smooth muscle [24]. Indeed, recent studies by Chiu et al. [25] in rat aorta as well as our earlier studies [21] indicate a good correlation between PI hydrolysis and intracellular calcium release. Furthermore, we found that levels of phosphatidylinositol-4,5-bisphosphate (PIP_2), the precursor to IP_3 , were decreased in aortae from septic rats [5]. Alterations in calcium mobilization from sarcoplasmic reticulum have also been reported in vascular smooth muscle during endotoxic shock. Soulsby et al. [26] showed that the ATP-dependent calcium uptake and calcium-stimulated ATPase activity were depressed by endotoxin in the microsomal-enriched fraction from canine aorta. Since the sarcoplasmic reticulum may be the major system controlling free cytoplasmic calcium in blood vessels [8,27], changes in the release and uptake of calcium from the sarcoplasmic reticulum could be a major factor in explaining the depressed contractility in septic aorta.

Overall, we have demonstrated striking alterations in aortic α_1 -adrenergic receptor-mediated signal transduction during sepsis. We previously showed a decrease in α_1 -

adrenergic receptors and in the α_1 -adrenergic receptor-mediated PI hydrolysis [5]. Furthermore, the decrease in calcium release from sarcoplasmic reticulum, as demonstrated by the decrease in calcium efflux, could be caused by a decrease in PIP_2 hydrolysis. α_1 -adrenergic receptor stimulation of calcium influx across the calcium channels in the plasmalemma was also depressed in sepsis. In addition, we have also shown an alteration of protein phosphorylation in aortae from septic rats [28] which may reflect changes in the activation, but not the amount, of protein kinase C. Thus these findings suggest that a portion of the diminished responsivity seen in rat aorta during sepsis could be related to perturbations in this receptor-mediated cascade.

Finally, it is important to realize that, similar to these findings in aorta, Spitzer's group found alterations in adrenergic and vasopressin receptor-mediated mobilization of intracellular calcium in hepatocytes [29]. Also, analogous changes in α_1 -adrenergic receptors were also observed in hepatocytes from endotoxemic rats [30] as well as nearly the same alterations in PI metabolism [31].

CONCLUSIONS

During experimental sepsis we found a depressed mobilization of extracellular calcium through calcium channels and depressed calcium efflux, indicating a diminished calcium release from the sarcoplasmic reticulum. The concentration of protein kinase C was not altered. These findings along with our previous results suggest an alteration in aortic α_1 -adrenergic receptor-mediated signal transduction in sepsis.

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The experiments described in this paper were performed in adherence to the NIH guidelines for the use of experimental animals.